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A New Enzyme Immunoassay for the Detection of Enteroviruses in Faecal Specimens

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A new enzyme immunoassay (EIA) for direct detection of enteroviruses based on a group-specific monoclonal antibody was evaluated using stool samples from patients with suspected enteroviral infection. The EIA was compared with polymerase chain reaction (PCR) and virus isolation in cell culture. Of 204 samples tested, 20 were positive by EIA, 34 by PCR, and 18 by cell culture. Compared with PCR, the most sensitive method, the sensitivity of EIA was 58% (20/34); the sensitivity of cell culture isolation was 52% (18/34). The results of both assays correlated in only 60% of cases. The combination of EIA and cell culture isolation detected 76% of PCR-positive stool samples. Enterovirus EIA provides results within 3–4 hr and requires only standard EIA equipment. It represents a rapid, reliable, and cost-effective diagnostic tool for enterovirus diagnosis from faecal samples. Negative results must be confirmed by other techniques, such as PCR or virus isolation in cell culture. ***J. Med. Virol. 60:439–445, 2000.***

KEY WORDS: enterovirus; direct detection of antigen; enzyme immunoassay; polymerase chain reaction; cell culture; virus isolation

INTRODUCTION

The genus enterovirus, which belongs to the family Picornaviridae, is an antigenically variable infectious agent that includes 67 recognized serotypes: poliovirus (three serotypes), coxsackievirus A (23 serotypes), coxsackievirus B (six serotypes), echovirus (31 serotypes), and the numbered enteroviruses 68 to 71. Most enteroviruses replicate initially in the gastrointestinal tract. The shedding of virus from oropharynx and especially in faeces can be massive over the course of several weeks. Generalisation of virus infection occurs either by direct hematogenous spread or by axonal transport from peripheral nerves.

Nonpoliovirus enterovirus infections are responsible for a wide variety of clinical syndromes. The most common infections in human beings are asymptomatic or produce a mild febrile respiratory illness. More severe courses of clinical infections result in meningitis, encephalitis, paralytic syndromes [Muir and van Loon, 1997; Zaotis and Klein, 1998], or acute and chronical myocarditis [Kandolf, 1996; Archard et al., 1998]. Enteroviruses can play a role in the viral triggering of autoimmune diseases, among them, diabetes mellitus [Roivainen et al., 1998] and Graves disease [Kraemer et al., 1998]. Intrauterine enteroviral infection can lead to adverse effects on the fetus [Palmer et al., 1997; Van den Veyver et al., 1998]. Neonates and children under 10 years account for 82–91% of all patients with nonpolio enteroviral isolates [Druyts-Voets, 1997; Moore, 1982]. Because infections caused by enteroviruses cannot be clinically distinguished from diseases caused by other viruses or bacteria, particularly in children and neonates, and because of possible therapeutic interventions by antipicornaviral agents (pleconaril) [Abdel-Rahman and Kearns, 1998], rapid and reliable diagnostic tests are needed in the near future.

Laboratory diagnosis of enteroviral infections is based on detection of virus in appropriate clinical specimens, such as throat swabs, nasopharyngeal secretions, rectal swabs, stool, vesicle fluid, and cerebrospinal fluid (CSF). Serologic investigations provide corroborating evidence of recent or acute enteroviral infections. The standard for the diagnosis of enteroviral infections is isolation of the virus in cell culture. However, some coxsackie A virus types can be propagated only in newborn mice. Typing of isolates is usually done by neutralisation assays with antisera (Lim Benyesh Melnick pool sera) or monoclonal antibodies. Another possibility for virological diagnosis is the detection of viral genome. Numerous reverse transcription–polymerase chain reaction (RT-PCR) methods capable of detecting RNA of most enterovirus serotypes

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have been developed. A simple and rapid semi-automated colorimetric PCR–enzyme immunoassay (EIA) also has been reported [Rotbart, 1991; Searle et al., 1997].

An alternative to RT-PCR and cell culture is the detection of viral antigens in clinical samples by enzyme immunoassay. The assay described here is based on a solid-phase bound anti-enterovirus-specific monoclonal antibody for direct capture of enteroviral antigen in clinical specimens. Captured virus particles are detected by a biotinylated monoclonal antibody, also specific to enteroviruses. Comparable EIAs were developed for the detection of herpes simplex virus, rotavirus, adenovirus, respiratory syncytial virus, and astrovirus [Pedneualt et al., 1994; Steele et al., 1994; Bryden and Bertrand, 1996; Shastri et al., 1998; Slomka et al., 1998].

MATERIALS AND METHODS

Virus Strains

Coxsackieviruses A5, A7, A10, A16 (CAV-5, -7, -10, and -16), and B1–B6 (CBV-1 to CBV-6); polioviruses 1–3 (PV-1 to PV-3); echoviruses 9, 11, and 20; herpes simplex virus type 1 and 2 (HSV-1 and HSV-2); and respiratory syncytial virus (RSV) adapted to tissue culture were originally obtained from different sources—the American Type Culture Collection (Rockville, MD) and Behringwerke (Marburg, Germany)—or isolated from clinical specimens using different cell culture systems. Viruses were propagated in human embryonic lung fibroblasts (HEL), primary green monkey kidney cells (PMK), and the human colon carcinoma cell line CaCo-2. Virus strains were used for antibody confirmation of the 9D5 monoclonal antibody (Chemicon, Temecula, CA; Virion, Switzerland) in order to test its specificity. Before testing, all enterovirus strains were typed by virus neutralisation using the Lim Benyesh Melnick antiserum pools supplied by the World Health Organizaton Reference Laboratory in Copenhagen.

Clinical Samples

Clinical specimens (stools and CSF) were obtained from practitioners and paediatric hospitals in Germany and tested as part of the daily diagnostic routine. They originated from patients who were suspected of having enteroviral infections on the basis of such illnesses as meningitis, encephalitis, or hand-foot-mouth disease or gastrointestinal symptoms. Partial information concerning the onset of illness was available for all patients. A total of 204 stool specimens from 204 patients, submitted to the laboratory between May 1 and October 31, 1998, were stored at –70°C until testing in the antigen EIA.

Virus Isolation

For virus isolation, 0.5–1 g of stool was suspended in 5 ml phosphate-buffered saline (PBS) and centrifuged for 5 min at 1,200*g*. The supernatant was sterile-filtered (0.2 μm), and 200 μl each of the suspension was inoculated in duplicate onto HEL, PMK, and CaCo-2 cells. Before testing, CaCo-2 cells were pre-incubated overnight in minimal essential medium with Earle's salts and nonessential amino acids with 4 μg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)–treated trypsin (Sigma, St. Louis, MO) and without fetal calf serum (FCS), in order to activate virus at the time of inoculation [Pinto et al., 1994]. Maintenance medium for CaCo-2 cells (minimum essential medium with Earle's salts (E-MEM) with antibiotics) also contained 4 μg/ml TPCK-treated trypsin. All tissue cultures were incubated at 37°C for up to 10 days and checked daily for cytopathic effects. The enteroviral cytopathic effects were confirmed by immunofluorescence antibody staining using the enterovirus screening set (Chemicon). Briefly, infected cells were harvested from cell culture tubes, and the cells were pelleted by centrifugation (5 min at 850*g*). The supernatant was discarded, and the cell pellet was resuspended in 100 μl PBS; 10 μl of the suspension was then spotted onto slides. The slides were air-dried and fixed for 10 min in ice-cold acetone; 10 μl of labelled antibody was added, and slides were incubated for 30 min at 37°C in a moist chamber. After washing with PBS, the slides were examined using a fluorescence microscope. Enterovirus-infected cells show a bright, spotty cytoplasmic fluorescence.

Isolation of Viral Nucleic Acid

Viral nucleic acid was extracted from CSF with the High Pure Viral RNA Kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. Stool samples were suspended in PBS and centrifuged, and 200 μl of supernatant was used for nucleic acid isolation. Samples of water were subjected to the same treatment as clinical samples, providing a control for contamination.

Primers and Reverse Transcription–Polymerase Chain Reaction

Primers and PCR conditions are described elsewhere [Searle et al., 1997]. In brief, primers for cDNA synthesis and PCR were designed to amplify a part of the highly conserved 5′ noncoding region of the enteroviral genome. The cDNA synthesis and PCR were carried out in 50-μl reaction volumes using Expand Reverse Transcriptase for RT and *Taq*-DNA-Polymerase for PCR (Roche Diagnostics).

Detection of Polymerase Chain Reaction Products

Detection of PCR products was carried out using a commercial PCR-EIA system (Roche Diagnostics). All steps, including DIG detection, washing, substrate incubation, and photometric measurement, were done at 37°C in the Behring EIA-Processor III (Behringwerke), according to the manufacturer's instructions. Precautions were taken to avoid the possibility of false-positive PCR results during nucleic acid isolation and subsequent PCR steps [Kwok and Higuchi, 1989].

Negative controls were included during sample preparation and in every amplification run.

Enzyme Immunoassay for Detection of Antigen

For EIA development, the Pan-Enterovirus Blend monoclonal antibody 9D5 (Chemicon, Virion) directed against the group-specific antigen of enteroviruses was used. The reactivity of the monoclonal antibody was described by Klespies et al. 1996. The monoclonal antibody was affinity-purified on a 1-ml protein A column (Pharmacia, Sweden). Microtiter plates (Maxisorp, Denmark) were precoated overnight at 4°C with 100 μl of purified monoclonal antibody (2 °g/ml) in 0.05 mol/L carbonate buffer, pH 9.2. Twenty percent of the stool suspensions were prepared using sample diluent containing 1% bovine serum albumin in PBS with 0.05% Tween 20; 100 μl of each sample was incubated for 60 min at 37°C. Afterward, plates were washed three times with wash solution (PBS with 0.05% Tween 20). Biotinylated 9D5 antibody was added to each well, followed immediately by avidin–horseradish peroxidase conjugate (Sigma). Biotinylation of purified monoclonal antibody was done with sulfosuccinimidyl-6-(biotinamido)hexanoate (EZ-Link-Sulfo-NHS-LC-Biotin) (Pierce, Rockford, IL). After incubation at 37°C for 30 min, plates were washed four times, and 3,3′, 5,5′-tetramethylbenzidine (TMB) substrate (Behringwerk) was added. The reaction was stopped with 50 μl 1 mol/L sulfonic acid, and absorbance was measured at 450 nm (A_{450}). The index was calculated as ratio A_{450} of patient's sample to A_{450} of negative control. The A_{450} value of the negative control was not higher than 150 optical density. As negative control, a mixture of 20 faecal samples from patients with excluded enterovirus infection was used. A sample was interpreted to show positive results if the calculated index was ≥ 2.

Enzyme Immunoassay for Detection of IgM Antibodies

Microtiter plates (Polysorp, Denmark) were coated overnight at 4°C with purified CBV-5 and echovirus 9, propagated in Vero cells, and with control antigen derived from noninfected Vero cells. Serum samples for testing, pretreated with rheumatoid factor absorbent (Behringwerke), were diluted 1:100 in PBS with 0.05% Tween 20, supplemented with 1% bovine serum albumin and applied in 100-μl aliquots to coated wells. Plates were incubated for 1 hr at 37°C. After washing three times in PBS with 0.05% Tween 20, rabbit anti-human IgM horseradish peroxidase conjugate (Behringwerk) was added to each well. After incubation at 37°C for 30 min, plates were washed four times, and TMB substrate was added. The reaction was stopped with 50 μl 1 mol/L sulfonic acid, and the A_{450} was measured. The IgM titre was determined as the difference between absorbance values of antigen and control antigen. Results were considered positive or negative with respect to the cutoff level. The cutoff was defined as the duplicate mean absorbance of 200 serum specimens from healthy persons.

Complement Fixation Test

The complement fixation (CF) test was carried out with picornavirus antigen (Virion, Germany). Single serum samples with CF titers of 1:128 and more were considered positive, without differentiation between acute and previous infection. A fourfold increase in CF titre was considered definitive etiologic evidence of acute enteroviral infection.

RESULTS

Clinical samples were obtained from 204 patients with suspected enteroviral infection. These 204 stool samples were simultaneously tested by three methods: isolation of virus in cell culture, detection of viral antigen by EIA, and detection of viral RNA by RT-PCR. One hundred and seventy probes were negative in all tests, and 34 probes were positive in at least one test. Table I presents the results for these positive samples. Overall, RT-PCR identified 34 enterovirus-positive samples. Positive RT-PCR results for 26 samples were confirmed by virus isolation in cell culture or antigen detection in EIA. Twelve of the RT-PCR-positive samples were positive by all three assays. Virus isolation was positive for 18, and antigen EIA was positive for 20 of the RT-PCR-positive samples. Eight samples were positive by RT-PCR only.

Tables II and III show a detailed analysis of the eight samples found to be positive only by RT-PCR. Table II describes three cases in which enteroviral infection was established by serology. In case 27, a 1-year-old baby with congenital cytomegalovirus infection in anamnesis was hospitalized with fever and meningitis. Nine days after onset, RT-PCR of CSF showed negative results. Only on the 17th day after onset did RT-PCR show positive results for enterovirus in stool and in CSF. Examination of the serum on the same day showed positive results on IgM EIA. The titre of CF antibodies was 1:128. After 5 months, stool and serum samples from this patient were examined again. All results were negative. In case 31, the enteroviral infection was also confirmed by detection of virus with RT-PCR in stool and CSF and increased antiviral CF and IgM antibodies in serum. In case 32, CSF was not available, but RT-PCR showed positive results in the stool sample collected 6 days after onset of symptoms. Acute enteroviral infection was confirmed by a fourfold increase in CF titre on serologic testing 10 days later.

In five cases, enterovirus infection could not be confirmed by serologic methods. These cases are shown in Table III. In cases 28 and 30, no CSF was available, and serum samples showed negative results, but stool specimens gave positive results by RT-PCR. In case number 29, RT-PCR showed positive findings in stool, but IgM antibodies were not detected, and the CF antibody titer was high (1:128) just on the second day from the onset and had not changed 1 month later. In case 33, hand-foot-mouth disease was diagnosed in a 2-year-old child. No vesicle fluid was available for virus detection. The stool sample showed positive results on

TABLE I. Clinical Data of 34 Patients With Positive Results for Enterovirus in Stool Samples by RT-PCR*

No.	Age	Symptoms	Day after onset	RT-PCR	Virus isolation	EIA	Index (EIA)[a]
1	8 years	Meningitis	4	+	+	+	30,0
2	10 years	Meningitis	1	+	+	+	14,0
3	8 years	Meningitis	1	+	+ CBV-2	+	23,0
4	7 years	Meningitis	n.k.	+	+ CBV-2	+	5,0
5	8 years	Fever, meningitis	3	+	+ CBV-2	+	3,4
6	11 years	Meningitis	n.k.	+	+ CBV-2	+	2,4
7	4 years	Meningitis	n.k.	+	+ Echo-11	+	2,3
8	10 years	Meningitis	2	+	+ Echo-30	+	2,0
9	9 years	Fever, meningitis	n.k.	+	+	+	2,6
10	4 days	n.k.	2	+	+ Echo-11	+	2,3
11	1 day	Meningitis	n.k.	+	+ Echo	+	2,0
12	4 years	Meningitis	4	+	+	+	2,0
13	30 days	Fever, meningitis	n.k.	+	–	+	2,0
14	32 years	Fever	9	+	–	+	10,0
15	5 years	Fever, meningitis	8	+	–	+	70,0
16	5 years	Meningitis	1	+	–	+	6,3
17	3 months	Mother had proven Coxsackievirus infection	n.k.	+	–	+	3,0
18	10 days	Viral infection	n.k.	+	–	+	2,0
19	1 year	Fever, viral infection	3	+	–	+	2,1
20	13 days	Mother had proven Coxsackivirus infection	n.k.	+	–	+	2,0
21	3 months	Meningitis	n.k.	+	+ Cox B1	–	0,9
22	1 year	Enteritis	n.k.	+	+	–	1,4
23	1 year	Enteritis	n.k.	+	+ Cox B5	–	0,7
24	2 months	n.k.	n.k.	+	+ Cox B5	–	1,4
25	3 months	Enteritis	n.k.	+	+	–	1,4
26	3 months	Enteritis	n.k.	+	+	–	1,4
27	1 year	Meningitis, enteritis	14	+	–	–	0,5
28	5 years	Meningitis	5	+	–	–	1,1
29	21 years	Fever, infection	30	+	–	–	1,4
30	12 years	Meningitis	6	+	–	–	1,4
31	6 years	Meningitis	6	+	–	–	1,3
32	24 years	Meningitis	n.k.	+	–	–	0,8
33	2 years	Hand-foot-mouth disease	8	+	–	–	1,2
34	26 years	Mother of child with hand-foot-mouth disease	10	+	–	–	0,9

RT-PCR, reverse transcription–polymerase chain reaction; EIA, enzyme immunoassay; n.k., not known.
*Comparison of virus detection by RT-PCR, virus isolation, and direct antigen detection.
[a]Index ≥2 is positive result.

RT-PCR, but virus could not be isolated in cell culture. The child's mother (case 34) was pregnant and had no symptoms of acute enteroviral infection and no increase in antiviral IgM antibodies in serum, despite the positive finding by RT-PCR in stool. In 18 stool specimens, enteroviruses were isolated in cell culture (Table I). In all of these samples, enteroviral RNA was also detected by RT-PCR, and antigen EIA showed positive results for 12 of 18 (66%) samples. Twenty samples were positive by direct antigen EIA (Table I). All of these samples showed positive findings on RT-PCR, but virus isolation in cell culture was positive for only 12 of 20 (60%) samples.

For the determination of test sensitivity, all 34 samples that were positive by RT-PCR were considered to be true positive. The 170 samples that were negative on all assays were considered to be true negative. Cell culture isolation sensitivity was 52% (18/34), and antigen detection EIA sensitivity was 58% (20/34). The EIA was not sensitive enough to detect enteroviruses in 10 RT-PCR-positive CSF samples. The results of sensitivity for the three assays are summarized in Table IV. Determination of the type-specific reactivity of EIA was made with supernatants from Vero cells infected with viruses CAV-5, -7, -10, and -16; CBV-1 to CBV-6; PV-1 to PV-3; echoviruses 9, 11, and 20; HSV-1 and -2; and RSV. Infectivity of the virus in supernatants was 10^5–10^7 tissue culture infective dose ($TCID_{50}$/ml). The test was positive for all enteroviruses and negative for HSV-1 and HSV-2 and for RSV.

DISCUSSION

Rapid and reliable diagnosis of enteroviral infection limits the use of antibiotics in patients with fever and meningitis and saves costs [Marshall et al., 1997]. To date, the diagnosis of enteroviral infections has been done only by exclusion. But the discovery of pleconaril, an orally active broad-spectrum antipicornaviral agent, opens perspectives for special target treatment of enterovirus infections [Abdel-Rahman and Kearns, 1998]. Therefore, rapid detection of enteroviruses becomes especially important for the clinical treatment of infected patients. The standard procedure for detection of enteroviruses is virus isolation in cell culture. This

TABLE II. Clinical Samples with Discrepant RT-PCR Results Compared With Virus Isolation in Cell Culture and Antigen Detection*

No.	Age (yr)	Date of onset	RT-PCR—stool	RT-PCR—CSF	Virus isolation—stool	Antigen EIA—stool	IgM EIA—serum	CF—serum	Clinical data
27	1	10.05.98		19.05.98 negative					Fever, meningitis (congenital CMV in anamnesis, on 19.05.98 CMV-PCR in CSF was neg)
			27.05.98 positive	27.05.98 positive	27.05.98 negative	27.05.98 negative	27.05.98 positive	27.05.98 1:128	
			28.09.98 negative		28.09.98 negative			28.09.98 1:16	Fever after vaccination (MMR and Hep-B)
31	6	14.08.98	14.08.98 positive	14.08.98 positive	14.08.98 negative	14.08.98 negative	14.08.98 positive	14.08.98 1:128	Meningitis
32	24	05.07.98	11.08.98 positive	n.d.	11.08.98 negative	11.08.98 negative	n.d.	11.08.98 1:32	Meningitis
							22.08.98 positive	22.08.98 1:128	

*RT-PCR, reverse transcription–polymerase chain reaction; CSF, cerebrospinal fluid; EIA, enzyme immunoassay; CF, complement fixation; CMV, cytomegalovirus; MMR, measles-mumps-rubella; Hep-B, hepatitis B; n.d., not determined. Cases with serologic confirmation of enterovirus infection.

TABLE III. Clinical Samples with Discrepant RT-PCR Results Compared With Virus Isolation in Cell Culture and Antigen Detection*

No.	Age (yr)	Date of onset	PCR—stool	PCR—CSF	Cell culture—stool	Antigen EIA—stool	IgM EIA—serum	CF—serum	Clinical data
28	5	08.08.98	10.08.98 positive	n.d.	10.08.98 negative	10.08.98 negative	10.08.98 negative	18.08.98 1:16	Meningitis
29	21	20.07.98	18.08.98 positive	n.d.	18.08.98 negative	18.08.98 negative	21.07.98 negative	21.07.98 1:128	22-week gestation, influenza-like illness, fever
							19.08.98 negative	19.08.98 1:128	
30	12	04.08.98	07.98.98 positive	n.d.	07.07.98 negative	07.07.98 negative	07.07.98 negative	07.07.98 1:32	Meningitis
33	2	09.08.98	17.08.98 positive	n.d.	17.08.98 negative	17.08.98 negative	n.d.	n.d.	Hand-foot-mouth disease
34	26	10.08.98		n.d.			10.08.98 negative	10.08.98 1:64	Mother of child (no. 33) with hand-foot-mouth disease 24-week gestation, asymptomatic
			17.08.98 positive		17.08.98 negative	17.08.98 negative	17.08.98 negative	17.08.98 1:64	

*RT-PCR, reverse transcription–polymerase chain reaction; CSF, cerebrospinal fluid; EIA, enzyme immunoassay; CF, complement fixation; n.d., not determined. Cases without serologic confirmation of enterovirus infection.

TABLE IV. Sensitivity of Three Assays: Isolation of Virus in Cell Culture, Detection of Viral Antigen by EIA, and Detection of Viral RNA by RT-PCR

Assay	Sensitivity
PCR	100% (34)
Antigen detection EIA	58% (20/34)
Cell culture	52% (18/34)

RT-PCR, reverse transcription–polymerase chain reaction; EIA, enzyme immunoassay.

method is labour-intensive and time-consuming. More rapid shell vial cultures lack sensitivity compared with conventional virus isolation [Van Doornum and De Jong, 1998].

RT-PCR can be used as an alternative rapid test for routine enterovirus diagnosis. RT-PCR is more sensitive than cell culture methods because of the possibility of detecting infectious and noninfectious virus particles; results can be obtained within 6 hr [Searle et al., 1997; Gorgievski-Hrisoho et al., 1998]. This method is also particularly useful for detection of enterovirus serotypes that cannot be propagated in cell culture. Furthermore, the use of standardised RT-PCR test components and automated or semi-automated procedures helps improve test reliability and rapidity. The occurrence of false-positive RT-PCR results, a major drawback in the past, can be considered significantly reduced today. Nevertheless, RT-PCR diagnostics require a special laboratory design.

An EIA was developed based on group-specific monoclonal antibodies. The EIA is easy to handle and rapid, and it can be done in every laboratory with standard EIA equipment. For development of the EIA, the group-specific monoclonal antibody 9D5 (Chemicon, Virion) was used. Another commercially available enterovirus group-specific monoclonal antibody, 5-D8/1 (Dako, Germany), was found to be less suitable for the test because of lower affinity. It is also possible to use a mixture of monoclonal antibodies against different enterovirus types instead of one monoclonal group-specific antibody. It should be noted that EIA is a very cost-effective diagnostic method, but group-specific and sensitive anti-enterovirus antibodies with high affinity are rare and relatively expensive.

The EIA showed broad group-specific reactivity with all tested enteroviruses and was negative with control samples containing HSV-1 and HSV-2 and RSV viruses. Klespies et al. [1996] showed that the 9D5 monoclonal antibody does not react with adenovirus, reovirus 2 and 3, hepatitis A virus, and HSV-1 and -2 but shows some cross-reactivity with rhinovirus. This cross-reactivity does not present a problem for testing of stool samples. All EIA-positive samples were retested by RT-PCR; positive results underscored the high specificity of the test. The clinical material best suited to EIA is stool, because stool samples contain enough virus for detection. In contrast, attempts to detect enterovirus in CSF by EIA failed. Other materials potentially containing enterovirus, such as throat swabs or vesicle fluids, were not tested.

The sensitivity of EIA (58%) is comparable to the sensitivity of cell culture (52%). A combination of EIA and cell culture detected 76% of RT-PCR-positive stool samples. The sensitivity was accounted on the basis of RT-PCR-positive results as "gold standard." The results of both assays correlated in only 60% of cases. This situation is similar to the problems encountered in the evaluation of direct antigen tests for other infectious agents, such as herpes simplex virus and respiratory syncytial virus [Dascal et al., 1989; Johnston and Siegel, 1990]. There are several theoretical explanations of discrepant results. For example, EIA can be positive and cell culture isolation negative because of shedding of noninfectious antigen or virus or loss of viral infectivity in samples. Moreover, some enteroviruses will not grow in standard tissue culture. Alternatively, cell culture isolation can be positive and EIA negative because of the greater sensitivity of virus isolation by in vitro amplification of infectious virus.

RT-PCR-positive results were considered true positive because of strict precautions to minimise the likelihood of false-positive results and because of the absence of positive results in the control group of patients who had infections caused by other agents. In eight cases of positive results by RT-PCR and negative results on both EIA and cell culture, additional information was obtained by RT-PCR in CSF, serologic tests, and clinical history. The positive RT-PCR results in stool in cases 27 and 31 were confirmed by positive RT-PCR results in CSF and by serologic determination of positive IgM antibodies. In case 32, paired serum samples were available. A fourfold increase in CF titre was taken as etiologic evidence of an enteroviral infection. In case 33, the clinical symptoms of hand-foot-mouth disease suggested the presence of enteroviral infection and explained the positive RT-PCR result in stool, though virus isolation and EIA showed negative findings. The child's mother (case 34) remained healthy despite a positive RT-PCR result in stool; no humoral answer on the infection was observed. CF titer and IgM antibodies were negative. In case number 29, the serologic test results suggest a previous rather than an acute enteroviral infection. The CF titre was high on the first day. The IgM titre was negative on the first day and remained constant for 1 month. The actual illness was most likely connected with another pathogen.

These data show that RT-PCR in stool samples can detect viral genome in patients who do not show signs of enterovirus-associated disease. Some asymptomatic individuals can shed a small quantity of virus for up to 4–8 weeks, which will be detected only by highly sensitive RT-PCR [Muir and van Loon, 1997]. The drawbacks of virus isolation in cell culture are well known. Isolation fails because of shedding of noninfectious virus and enteroviruses that do not grow in standard tissue culture. Furthermore, cell culture is laborious and time-consuming. EIA provides results in just 3–4 hr and requires only standard EIA equipment. This

assay is especially useful for small laboratories that cannot perform RT-PCR.

Virus can be detected in stool, throat, vesicle fluid, blood, and CSF. Stool samples are used most often for virus detection, because they are readily available. Moreover, serum samples are the most common clinical materials in enterovirus diagnosis, often in association with stool samples. The usefulness of faecal samples for enterovirus diagnosis in patients with aseptic meningitis has been established [Glimaker et al., 1992], but only virus detection in CSF is evidence of an enterovirus-associated disease. A positive result by EIA in stool is indicative of an enterovirus infection. The combination of antigen detection in stool and detection of IgM antibodies in serum may be a helpful strategy for diagnosis of enterovirus-associated diseases. Negative enterovirus EIA results in stool require confirmation by RT-PCR or isolation of virus in cell culture. The enterovirus EIA is a useful step in the diagnosis of enterovirus infections. A positive test result makes other tests, such as RT-PCR or virus isolation, superfluous and makes enterovirus diagnosis more rapid, easy to perform, and cost-effective.

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